ZOOLOGICAL SCIENCE 7: 287–295 (1990)

Formation of Ganglions and Stomodaeum in Normal and Separate Embryos of Horseshoe Crab, *Tachypleus tridentatus*

Tomio Itow, Tatsuya Masuda and Koichi Sekiguchi[1]

Department of Biology, Faculty of Education, Shizuoka University, Shizuoka 422, and [1]Jobu University, Shin-Machi, Gunma 370-13, Japan

ABSTRACT—The process of formation of ganglions and stomodaeum was examined in normal embryos and separate embryos whose stomodaea did not pass through the middle of the nervous system. The following facts were established. 1) The structure of the stomodaeum is recognizable aftre stage 14. 2) The cell masses of ganglions are observed clearly after stage 16; the commissures are stained by eosin after stage 19. 3) The final number of ganglions is settled by stage 19; this means that the formation of segment primordia is settled by stage 19. 4) The stomodaeum passes through the area between the commissure of the first prosomatic ganglions (chelicera segment) and that of the second ganglions. 5) The crossing of the stomodaeum and the nervous system is thought to be formed at stage 18–19. 6) The crossing is constructed during the clustering of the cells composing the brain; the stomodaeum does not determine the pathway for the clustering of these cells. 7) The position of the mouth is not appropriate as an indicator for determination of homologous segments among arthropodan species. 8) The crossing does not constitute a valid reason for rejecting the idea that Deuterostomia have originated from Protostomia.

INTRODUCTION

The morphology and embryology of the horseshoe crab have been described by many authors [*Tachypleus tridentatus*: 1–3; *Limulus polyphemus*: 4–7]. However, not all structures and processes have yet been clarified. Study of the formation of ganglions and stomodaeum, especially the crossing between the nervous system and the stomodaeum, remains incomplete. The present study tries to make this process clear for the Japanese horseshoe crab, *Tachypleus tridentatus*. For this purpose, we examined the formation of ganglions and the stomodaeum in normal embryos. We also examined the process in separate embryos whose stomodaea did not pass through the middle of the nervous system. Such embryos are formed by treatment with calcium-free seawater or DNA synthesis inhibitors [8, 9].

The cause of release of the crossing of the nervous system and stomodaeum is discussed in the light of cell construction during the process of embryonic development. As the crossing is a remarkable characteristic of Protostomia, the possibility for change of form and structure in macroevolution is also considered.

Accepted April 28, 1989
Received February 22, 1989

MATERIALS AND METHODS

Adult Japanese horseshoe crabs, *Tachypleus tridentatus* (Chelicerata, Arthropoda), collected in north Kyushu, Japan, were brought to Shizuoka University, where eggs were inseminated artificially. The developmental stages of the embryos were determined from Sekiguchi's normal table [2].

The separate embryos were obtained by 24-hr treatment with calcium-free seawater, 10^{-1} M $NaHCO_3$ or inhibitors of DNA synthesis (10^{-2} and 5×10^{-2} M hydroxyurea and 10–25 μg/ml azaserine). The treatment stages are described in the results.

Normal embryos and ones given these chemical treatments were stained vitally with neutral red and observed under a stereomicroscope. Normal and treated embryos were also fixed in Bouin's,

Carnoy's, or FAA (formalin-70% ethanol-acetic acid, 5 : 15 : 1) solutions, embedded in celloidin and paraffin, and sectioned at 5–20 μm. The sections were stained with Mayer's hematoxylin and eosin. Some normal embryos and larvae were dissected for an examination of the nervous system and alimentary canal.

RESULTS

Formation of ganglions and stomodaeum in normal embryos

In this paper, the ganglions in the front area of the 1st prosomatic segment (except for ganglions of the 1st segment) are called the brain.

Enlargement of the germ disc of the horseshoe crab finished at stage 10 (stage of completion of germ disc). Obvious morphogenetic movement started at stage 10. Observation with time-lapse cinemicrography in the previous study [10] had shown that the formation of the stomodaeum begins at the stage of morphogenetic movement (stage 11). The stomodaeum appeared at the anterior margin of the embryonic area. The position of the stomodaeum differed from that of the blastopore. In this process, two narrow bands are formed along the median body axis; these narrow bands may be early nervous systems.

At the stage of the appearance of prosomatic appendages (stage 14), the stomodaeum was observed as a tubular structure. The existence of neuroblasts which would become the brain was recognized, but construction of the brain was not yet complete (Fig. 1).

Ganglions could be observed in embryos fixed at the stage of development of prosomatic appendages (stage 16). The opening of the stomodaeum (mouth) could be observed clearly at the area in front of the 1st prosomatic segment (segment with chelicera=1st prosomatic appendages). However the development of the brain and other ganglions was incomplete, the commissures of ganglions in particular being under-developed. The formation of the epithelium of the stomodaeum proceeded further at stage 16.

When embryos at the stage after the 1st embryonic moulting (stage 18) were stained with neutral red, nervous systems were stained clearly. A brain and 9 pairs of ganglions could be observed. The commissures of the ganglions were not clear in the stained embryos or sectioned specimens, although they were recognized in fixed embryos. The mouth began to migrate posteriorly at stage 18; it was situated in the region of the 1st prosomatic segment at this stage.

At the stage after the 2nd embryonic moulting (stage 19), the commissures became clear. The neurofibers developed and were stained easily with eosin. The mouth migrated to the region of the 3rd prosomatic segment. The stomodaeum passed through the area between the commissure of the 1st prosomatic segment and that of the 2nd one.

All the ganglions were formed by stage 19; that is, the formation of all segment primordia was complete at this stage. The mouth was situated in

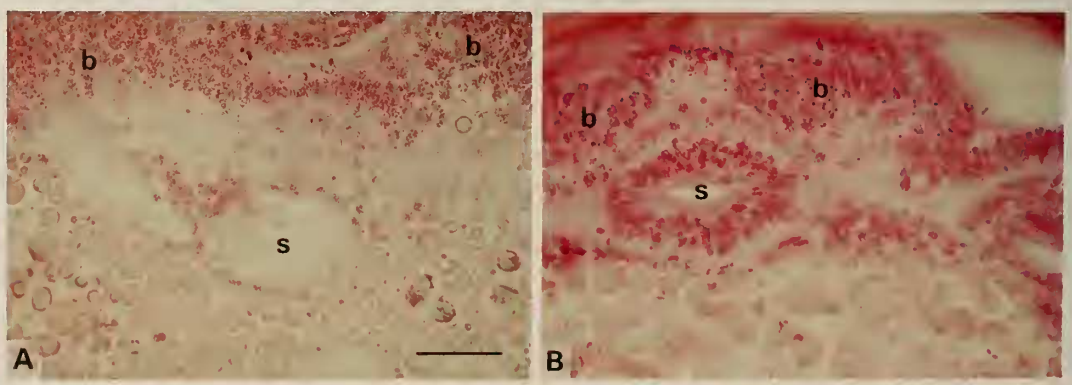

Fig. 1. The histological features in the neighboring region of the stomodaeum. A. Stage 14. B. Stage 16. b; prospective region of the brain, s; stomodaea. The bar shows 0.05 mm.

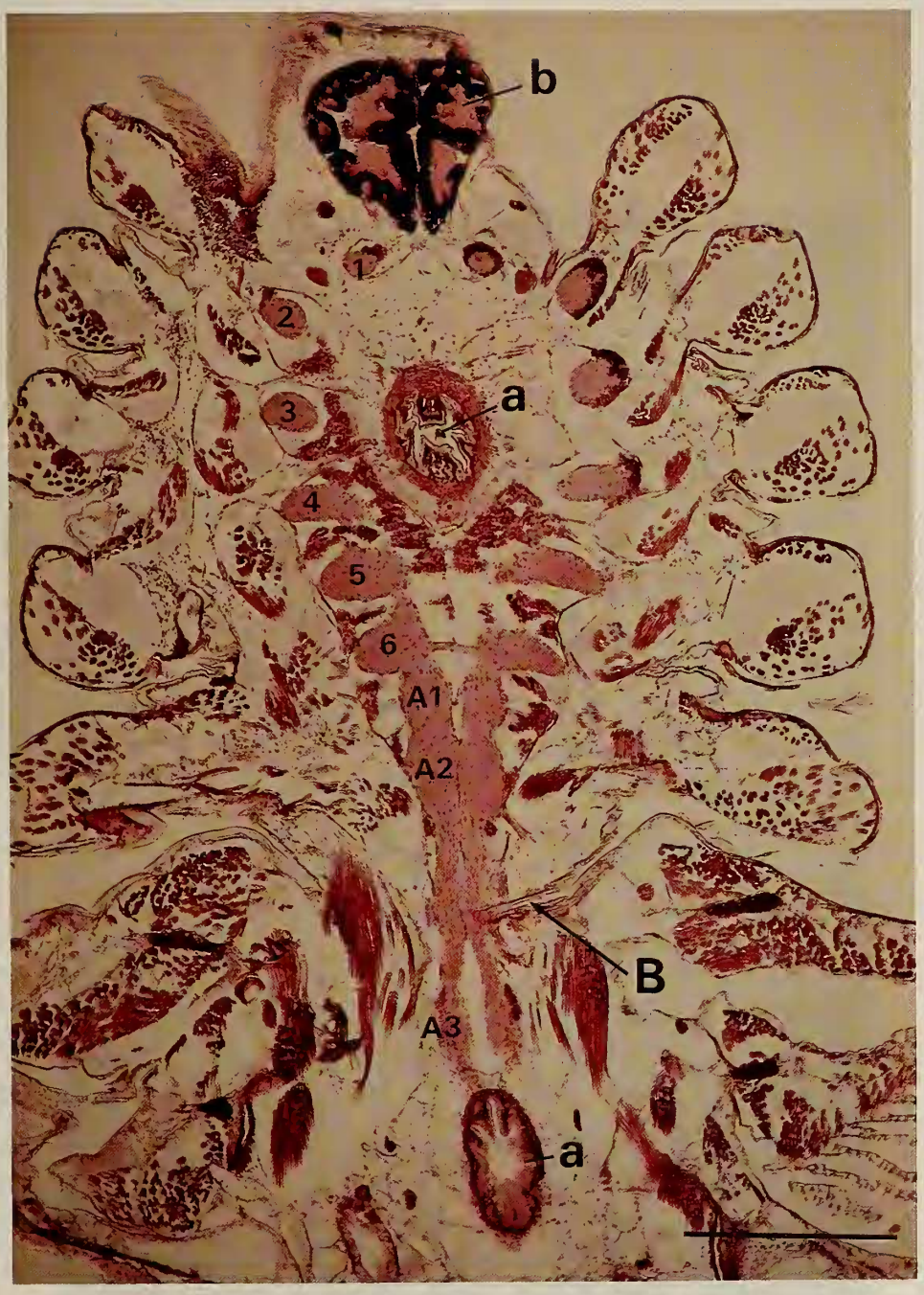

FIG. 2. The horizontal section of a 2nd instar larva. a; alimentary canal, b; brain, 1–6; each cephalothoracic ganglion, A1-A3; each abdominal ganglion, B; border between prosome and opisthosome. The number of pairs of prosomatic ganglions except for the brain is eight. The bar shows 0.5 mm.

the region between the 3rd and 4th prosomatic segments. The position was the same as that in the adults.

The embryos at stage 21 (the stage after the 4th embryonic moulting) have the same form and structure as the 1st instar larvae. At this stage the circumbuccal part (the structure of the peristome) was formed completely. The stomodaeum was well developed and differentiated. As a result, the proventriculus (fore-gut) and intestine (mid-gut) were also differentiated. However, there was yolk in the intestine, and the formation of the

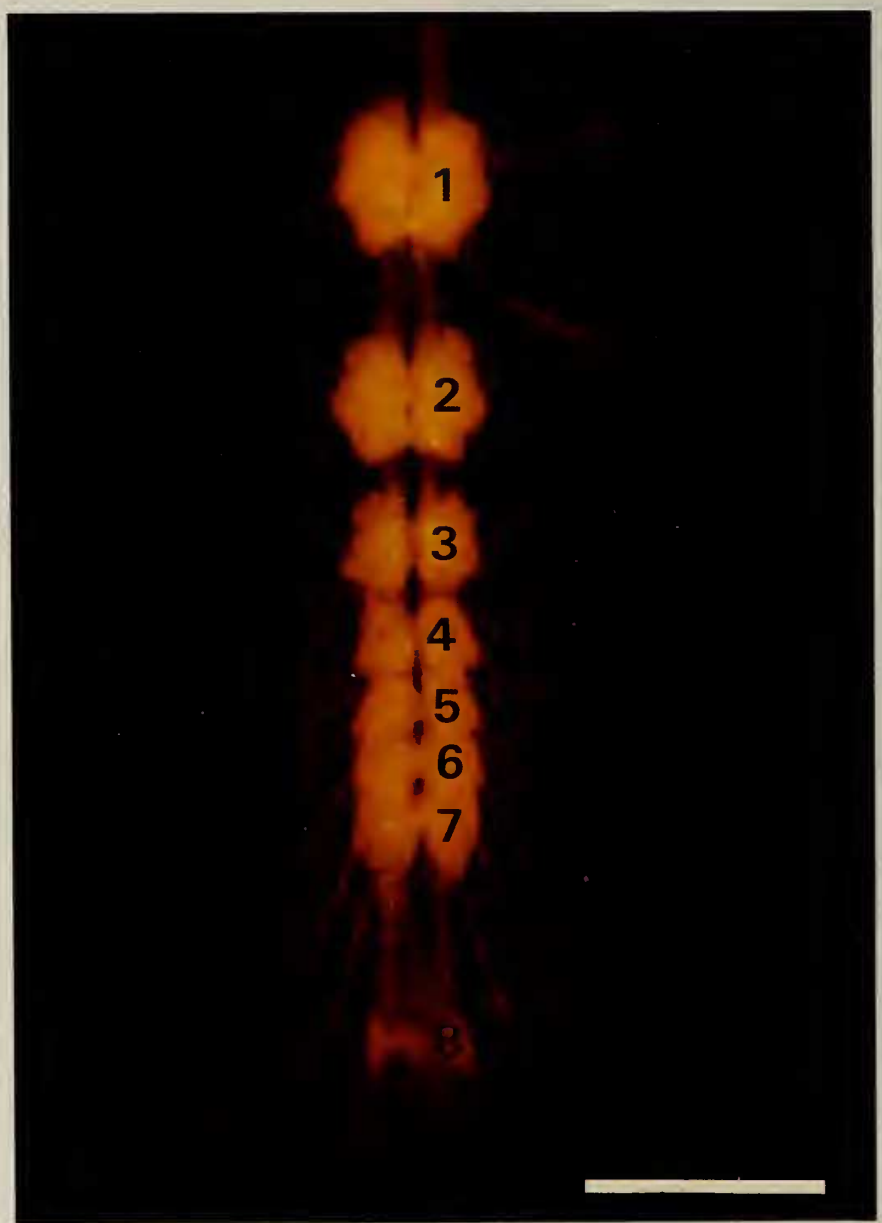

Fig. 3. The opisthosomatic nervous system of the 2nd instar larva. 1–8; each opisthosomatic ganglion. The 1st and 2nd abdominal ganglions belong to the prosome. The 1st opisthosomatic ganglion is equal to the 3rd abdominal one. The bar shows 0.5 mm.

alimentary canal was not complete.

The condition and position of the brain and other ganglions of the 1st instar larvae were similar to those of the 2nd instar larvae. In these larvae all the prosomatic ganglions had commissures. In the border area between the prosome and opisthosome there were no ganglions without appendages (Fig. 2). The prosome had a brain and 8 pairs of prosomatic ganglions. The prosomatic ganglions consisted of 6 pairs of cephalothoracic ganglions and 2 pairs of abdominal ganglions (ganglions of chilaria and operculum). The shape of the cephalothoracic ganglions differed from that of the abdominal ones. The opisthosome had 8 pairs of ganglions (the rest were abdominal ganglions) (Fig. 3). The shapes of the opisthosomatic ganglions were similar to those of the abdominal ganglions in the prosome.

The alimentary canal of the 2nd instar larva was complete. At this stage, formation of intestine was finished and the larvae began to eat.

Release of the crossing of the nervous system and stomodaeum in separate embryos

Calcium-free seawater, $NaHCO_3$ and inhibitors of DNA synthesis induced the separate embryo (Table 1, Fig. 4). The ventral plate of the separate embryo was divided into an anterior region and a posterior region.

The conditions of induction were as follows. When embryos were treated for 24 hr with calcium-free seawater or $NaHCO_3$ at stages 7, 8 and 9 (stage of enlargement of the germ disc, the gastrula stage), they developed into the separate embryos whose ventral plates were separated mainly at the region between the 3rd and 5th prosomatic segments. When treated for 24 hr at stages 10 and 11 (stages of obvious morphogenetic movement), the ventral plates of the treated embryos were separated mainly at the 2nd and 3rd prosomatic segments. Following treatment for 24 hr with an inhibitor of DNA synthesis at the stage of enlargement of germ disc, the treated embryos developed into separate embryos, whose ventral plates were separated mainly at the 2nd and 3rd prosomatic segments.

When embryos at the stage of enlargement of the germ disc were treated with calcium-free seawater or $NaHCO_3$, the connection between cells composing the germ disc was weakened. Their ventral plates were separated mainly at the region between the 3rd and 5th segments of the cephalothorax, which was formed in the process of enlargement of the germ disc. When embryos at the stage of enlargement of the germ disc were treated by inhibitors of DNA synthesis, cell proliferation of germ disc became incomplete and the cell density of the germ disc became low in spite of normal spreading of the germ disc. The incomplete germ disc was separated mainly at the 2nd and 3rd prosomatic segments in the process of obvious morphogenetic movement. During the movement, the embryonic area elongated anteriorly and posteriorly at the region where the 2nd and 3rd prosomatic segments had recently been formed. Calcium-free seawater and $NaHCO_3$

Table 1. The frequency of formation of separate embryos. The embryos were treated for 24 hr either at the stage of enlargement of the germ disc (I) or at the stage of obvious morphogenetic movement (II)

		Separate embryo Number (%)	Developed embryo Number (%)
[I]	Hydroxyurea 5×10^{-2} M	134 (32.1)	318 (74.2)
	Azaserine 25 μg/ml	73 (16.7)	436 (68.4)
[II]	$NaHCO_3$ 10^{-1} M	20 (19.2)	104 (13.4)
	Ca^{2+}-free seawater	57 (29.2)	192 (43.3)
	Normal seawater	6 (0.1)	6,829 (100.0)

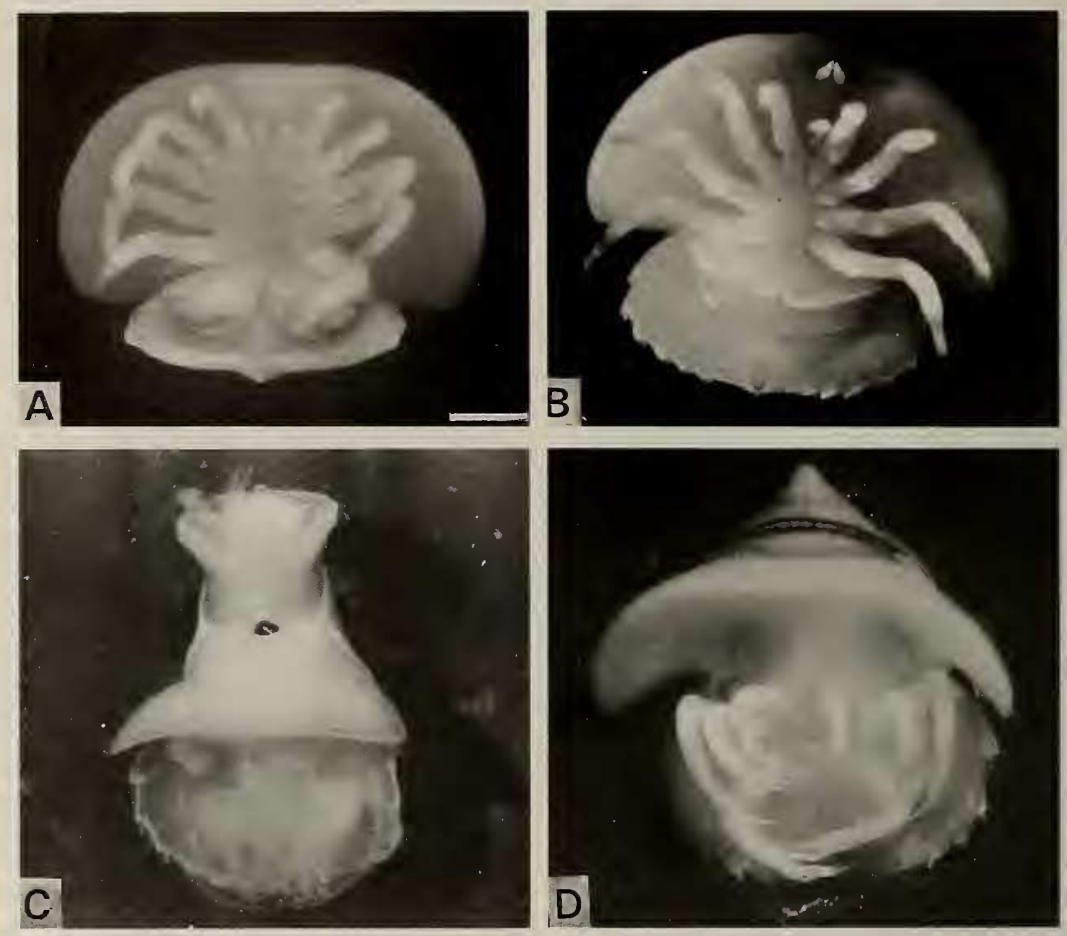

Fig. 4. Examples of a normal embryo and separate embryos. A: A normal embryo (stage 20). B: An incomplete separate embryo (stage 20) whose ventral plate is separated at the point between the 1st prosomatic segment and the 2nd one. C: A complete separate embryo (stage 21) whose ventral plate is separated at the point between the 3rd prosomatic segment and the 4th one. D: A complete separate embryo (stage 21) whose ventral plate is separated at the 2nd prosomatic segment. The 2nd appendages are lost. The bar shows 0.5 mm.

directly affected the central region of elongation at the stage of obvious morphogenetic movement. The ventral plates of the treated embryos were separated mainly at the 2nd and 3rd prosomatic segments.

The form and structure of the separate embryos depended on the position of separation. It was impossible to recognize any difference between embryos induced by different reagents, if the position of separation was the same. In addition the separate embryos obtained in normal seawater had the same characteristics as those of the separate embryos obtained by treatment with chemical reagents.

The separate embryos could be classified into the complete and incomplete separate ones. The ventral plate of the complete separate embryo was separated completely, but that of the incomplete one was not. The central nervous systems of both types of separate embryos were separated at the position of separation of the ventral plate, although light microscopy did not reveal clearly the very fine nervous fibers. On the other hand, alimentary canals were not separated in any type of embryo.

In some of the separate embryos, the stomodaea passed through the middle of the brain. The crossing of the nervous system and stomodaeum

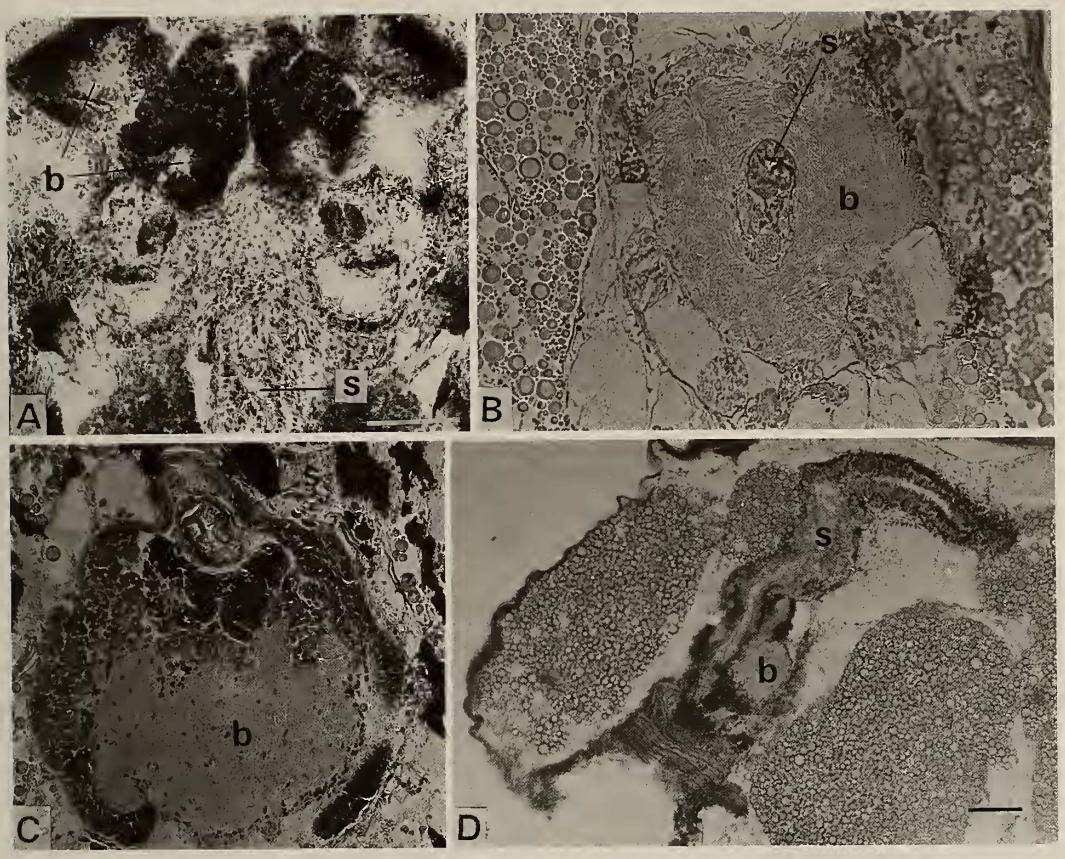

Fig. 5. The brains and stomodaea in a normal embryo and in separate ones at stage 20. A: A horizontal section of a normal embryo. B: A horizontal section of a separate embryo whose ventral plate is separated at the 2nd prosomatic segment. The stomodaeum passes through the middle of the brain. C: A horizontal section of a separate embryo whose ventral plate is separated at the region between the 1st prosomatic segment and the 2nd one. The stomodaeum and the nervous system do not cross each other. D: A longitudinal section of a separate embryo whose ventral plate is separated at the same region as in "C". "B" and "C" were taken at the same magnification. The bars indicte 0.1 mm.

was released in some of the separate embryos (Fig. 5). Release of the crossing was observed in both types of separate embryo, the complete separate embryos and the incomplete ones. This means that the release of the crossing was not related directly to the degree of separation, but it was related closely with the position of separation, that is, the extent of the anterior region of the separate ventral plate (Fig. 6). When the anterior region had more than three pairs of appendages, the stomodaeum passed behind the brain and both structures crossed each other. When it was moderate, the stomodaeum passed the middle of the brain. When the anterior ventral plate of the separate embryos had no or only one pair of cephalothoracic appendages, the crossing was released. The size of the posterior region of the separate ventral plate was not related directly to the release of the crossing of the nervous system and stomodaeum.

In embryos which had lost the anterior ventral plates (no-anterior embryos), there was no brain or stomodaeum. In the separate embryos whose ventral plates were separated at the 1st prosomatic segment, the neuroblasts of the brain were clustered behind the stomodaeum and formed the brain at this position. Crossign of the nervous system and stomodaeum did not occur (Fig. 7).

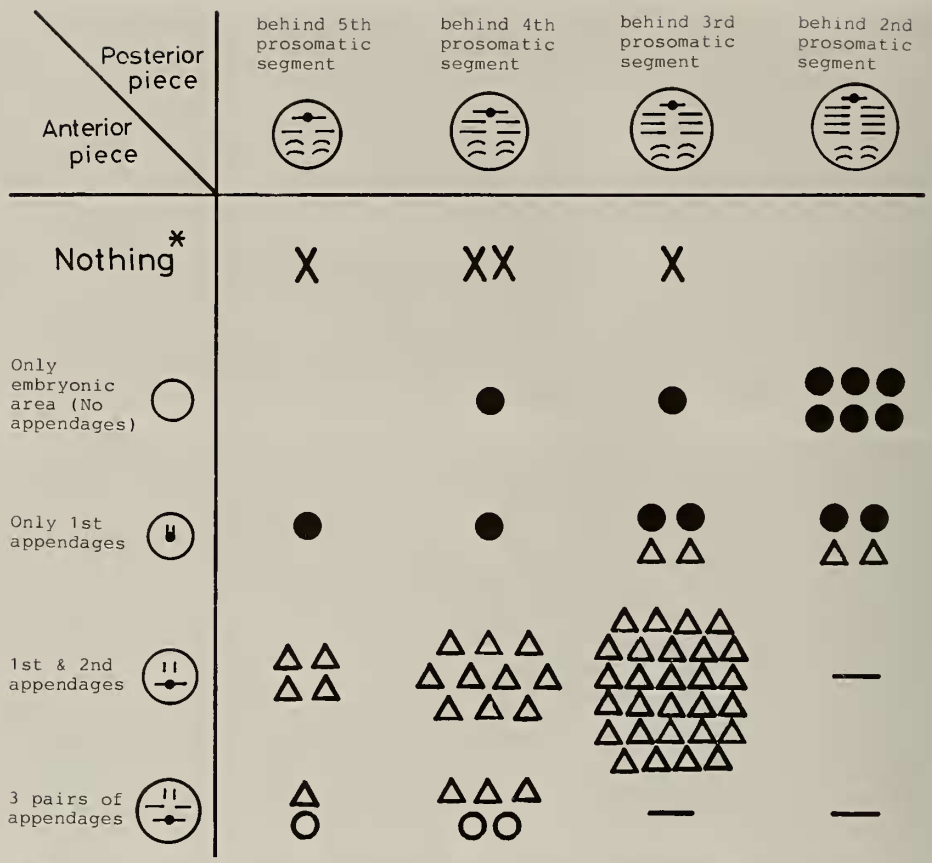

FIG. 6. Release of the crossing of the stomodaeum and nervous system. The schematic diagrams show the features of each piece of the separate ventral plates. Each mark indicates an embryo. ×: Embryos having no brains or stomodaea. ●: Nervous systems and stomodaea do not cross each other. △: Embryos whose stomodaea pass through the middle of the brain. ○: Embryos whose stomodaea pass behind the brain (normal position). *: Embryos without anterior pieces of separated ventral plates (=no-anterior embryos).

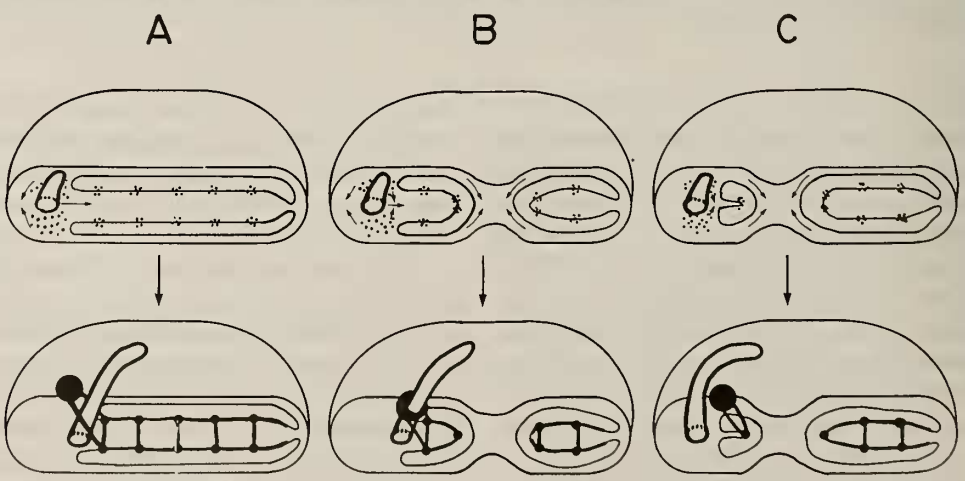

FIG. 7. Diagrammatic representations of development of normal embryos and separate embryos. A: A normal embryo. B: A separate embryo whose ventral plate is separated at the 2nd and the 3rd prosomatic segment. C: A separate embryo whose embryonic area is separated at the 1st prosomatic segment.

DISCUSSION

Several facts about normal embryos are recorded for the first time here. We consider the crossing of the nervous system and stomodaeum.

The tubular structure of the stomodaeum was formed at stage 14. The commissures of the ganglions were not formed at this stage. Remarkably developed commissures were recognizable at stage 19. The opening of the stomodaeum (mouth) first appeared in front of the 1st prosomatic segment. The mouth began to migrate posteriorly at stage 18. These results and the fact that the stomodaeum passed through the area between the commissure of the ganglions of the 1st and 2nd prosomatic segment indicate that the crossing of the nervous, system and stomodaeum occurs at stage 18–19.

Prospective cells of the stomodaea and brains were determined before the time of separation of the ventral plates, because regulation did not occur after separation. The prospetive cells of the brain were moved by the separation (Fig. 7). When the anterior part of the separate ventral plates was very small, the prospective cells of the brain were situated behind the stomodaeum. As a result the crossing of the nervous system and stomodaeum did not occur.

The release of crossing reveals the following four points. (1) The crossing is constructed through the process of clustering of brain cells. (2) It is not determined that the cells of the brain cluster anterior to the stomodaeum; that is, the stomodaeum does not determine the clustering point of the brain. Further the brain does not determine the pathway of elongation of the stomodaeum. (3) As the position of the mouth is changed easily, it is not appropriate as an indicator of determination of homologous segments among arthropod species [11, 12]. (4) The crossing is susceptible to modification in at least one representative Protostomia, the horseshoe crab. Attempts to derive Deuterostomia from Protostomia cannot be rejected because of the crossing of the alimentary canal and central nervous system.

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